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Interaction of triazolobenzodiazepines with benzodiazepine receptors

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Investigations of the biochemical properties of benzodiazepine receptors and screening of benzodiazepines utilize in-vitro [^{3}H]flunitrazepam ([^{3}H]FNZ) binding assays because of their simplicity, sensitivity and specificity. In previous in-vitro [^{3}H]FNZ binding studies, it has been demonstrated that triazolobenzodiazepines like alprazolam and triazolam have a high affinity for binding to benzodiazepine receptors (Sethy & Harris 1982). The present study describes some of the pharmacological characteristics of triazolobenzodiazepines revealed by using both in-vitro and ex-vivo [^{3}H]FNZ binding assays. The results of these assays have been compared with the in-vivo ED50's (obtained from published reports from our laboratories) of the compounds required to protect mice against leptazol(metrazol, pentylenetetrazol) and nicotine-induced seizures and against hypoxic stress.

Methods

In-vitro [^{3}H]FNZ binding to crude mouse brain membrane preparation for the determination of inhibition constant (K_i) of diazepam and triazolobenzodiazepines was carried out by the method of Sethy & Harris (1982). Ex-vivo [^{3}H]FNZ binding assays were carried out by the method described by Sethy et al (1983).

Male albino CF-1 mice bred at The Upjohn Company were kept under constant temperature conditions and diurnal lighting before use and all were killed at approximately the same time of day.

Alprazolam, diazepam, triazolam and U-43,465F (8-chloro-1-(2-dimethylamino)ethyl)-6-phenyl-4H-s triazolo(4,3-a)(1,4)benzodiazepine, *p*-toluene sulfonate) were dissolved in 2% ethanol. Adinazolam was dissolved in 0·9% sodium chloride solution. All drugs were administered by the intravenous route. Control mice received equal volumes (1 ml/100 g) of the vehicle. Animals were killed at 1, 3, 10, 30 and 60 min after the administration of drug. Whole brains minus cerebella were quickly removed and homogenized in 50 volumes of cold (4 °C) 50 mM Tris-HCl buffer, pH 7·4, using a Brinkman polytron, PCU-2-110 homogenizer for 30 s at setting 6. [^{3}H]FNZ binding was measured by incubating 1·0 ml aliquots of the homogenate with 0·1 ml of [^{3}H]FNZ (specific activity 84·8 Ci mmol^{-1}, NEN, Boston, Mass.) to give a final concentration of 0·7 nM. 0·1 ml of distilled water, or flurazepam (10 μM) and 0·8 ml of 50 mM Tris-HCl buffer, pH 7·4, to give a final volume of 2·0 ml. The mixture was incubated for 30 min at 25 °C and then filtered under vacuum through a Whatman GF/B filter. The incubation tube was rinsed with 5·0 ml of ice-cold buffer, and this rinse was applied to the filter. The filter was then washed three times with 5·0 ml aliquots of buffer. Finally the filter paper was placed in a scintillation vial containing 15 ml of Amersham ACS cocktail and the radioactivity was counted by a liquid scintillation spectrometer.

Specific binding was defined as total binding minus binding in the presence of 10 μM flurazepam. Specific binding represented over 95% of total binding. The results are expressed as [3H]FNZ bound: fmol mg^{-1} tissue. It was assumed in this study that ex-vivo binding of [3H]FNZ was inversely related to the concentration of parent drug and its active metabolites in the brain.

The relative potency of diazepam and triazolobenzodiazepines for inhibiting [3H]FNZ binding in-vitro is shown in Table 1. Triazolam was found to be the most potent compound (K_i 0·5 nM), whereas adinazolam was the least effective (K_i 88·3 nM). The in-vitro binding affinities (K_i) correlated with in-vivo ED50's for protecting mice against leptazol- and nicotine-induced seizures and against hypoxic stress, with the exception of U-43,465F (Table 1). This discrepancy in K_i (7·5 nM)

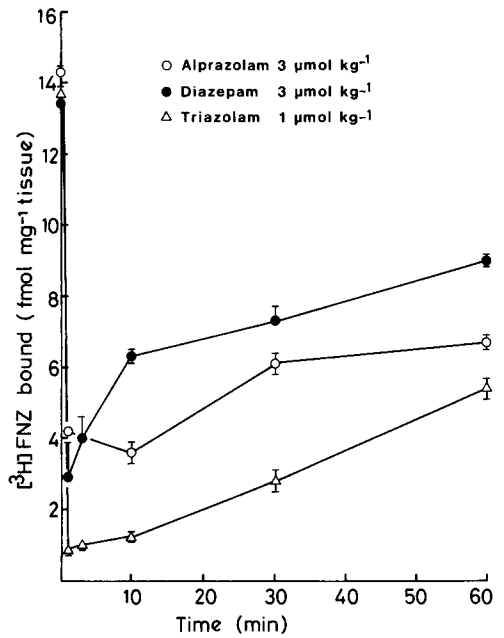

FIG. 1. [^{3}H]FNZ binding at various time intervals after i.v. administration of benzodiazepines to mice. Each point is the mean ± s.e. of four determinations.

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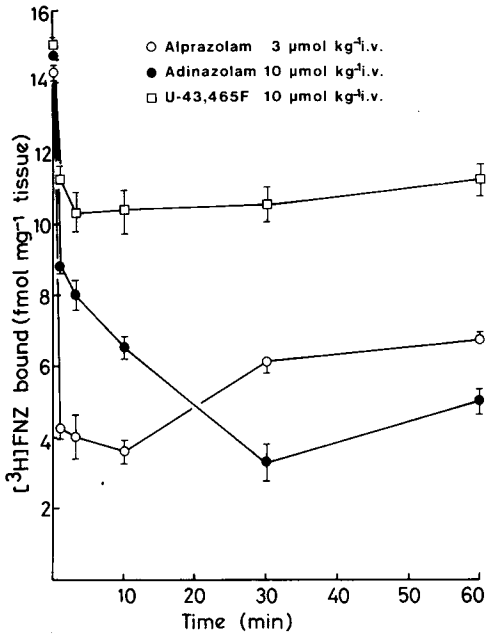


FIG. 2. [³H]FNZ binding at various time intervals after i.v. administration of triazolobenzodiazepines to mice. Each point is the mean ± s.e. of four determinations.

and ED50's against leptazol (25 mg kg^{-1})—and nicotine (2·8 mg kg^{-1})-induced seizures, and against hypoxic stress (20 mg kg^{-1}) with U-43,465F indicates that either this drug is metabolized at a rapid rate after i.v. administration or it is not passing into the brain adequately. The latter aspect has been investigated with U-43,465F and with other benzodiazepines by using ex-vivo binding assays.

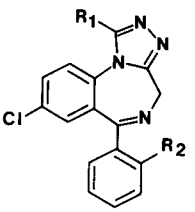
Triazolam, at dose of 1 μmol kg^{-1}, time-dependently decreased binding. The peak effect was achieved within 1 min after the i.v. administration of drug, and that effect persisted for 10 min. Inhibition of binding at 30 and 60 min was significantly ($P < 0·01$) less compared with that at 1, 3 and 10 min. Alprazolam (3 μmol kg^{-1}) had a profile similar to triazolam in an ex-vivo binding assay. Diazepam (3 μmol kg^{-1}) produced significant inhibition of binding 1 min after i.v. administration. The effect of diazepam on ex-vivo [3H]FNZ binding decreased significantly ($P < 0·01$) with each time investigated (Fig. 1).

The peak effect of adinazolam (10 μmol kg^{-1}) was observed at 30 min after i.v. administration of drug and significant inhibition of binding persisted at 60 min. In comparison with alprazolam and adinazolam, U-43,465F (10 μmol kg^{-1}) was significantly less potent in inhibiting ex-vivo [3H]FNZ binding. Also there was no time-dependent effect on [3H]FNZ binding after i.v. administration of U-43,465F (Fig. 2).

Alprazolam, diazepam, triazolam and adinazolam have a high affinity for benzodiazepine receptors, and are very potent in protecting mice against leptazol- and nicotine-induced seizures and against hypoxic stress (Table 1). The peak concentration of alprazolam, diazepam, triazolam and their respective metabolites in the brain is achieved within 1 min of intravenous administration (Fig. 1), indicating that these drugs may pass the blood-brain barrier rapidly. Adinazolam seems to have relatively slower penetration through the barrier as indicated by achievement of peak concentration at 30 min after administration of drug (Fig. 2).

U-43,465F has about one-half the affinity for benzodiazepine receptors in in-vitro [3H]FNZ binding assays compared with alprazolam. However, in in-vivo tests this compound is about 29–100 times less potent than

Table 1. Effect of diazepam and triazolobenzodiazepines on [³H]flunitrazepam binding to crude mouse brain membrane preparation in-vitro and its correlation with in-vivo pharmacological tests.



Drug*	R_1	R_2	K_i nM	ED50: μmol kg^{-1} i.p. Leptazol	Nicotine	Hypoxic stress
Diazepam	—	—	7·6	2·4	0·39	0·70
Triazolam	Me	Cl	0·5	0·03	0·01	0·10
Alprazolam	Me	—	3·4	0·42	0·18	0·22
Adinzolam	Me, Me>N-CH_2	—	88·3	2·0	0·24	11·16
U-43,465F	Me, Me>N-CH_2CH_2	—	7·5	46·46	5·20	37·17

* In-vivo data for diazepam, triazolam and alprazolam were obtained from Sethy & Harris (1982) 34: 115 and for adinazolam and U-43,465F from Hester et al (1980).

alprazolam. This lack of correlation between in-vitro and in-vivo tests with U-43,465F may be due to: (1) rapid metabolism to a compound with a low affinity to benzodiazepine receptors or (2) its poor penetration into the brain or both. The result of the present investigation indicates that U-43,465F did not achieve adequate concentration in the brain.

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Dependence of drug-protein binding parameters on human serum and albumin concentration

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As early as 1949, Klotz & Urquhart observed a large decrease in the absolute amount of methyl orange bound to bovine serum albumin when the protein concentration was increased five-fold, from 2 g to 10 g litre^{-1}. Thus, the ratios dye bound/total protein are smaller for 10 than 2 g litre^{-1} protein at a given concentration of free dye. Since then, many workers have reported on the artificial (Crooks & Brown 1973) or real dependence of the binding parameters of chemically highly diverse drugs on protein concentration (Brunkhorst & Hess 1965; Westphal 1971; Shen & Gibaldi 1974; Miese et al 1978; Bowmer & Lindup 1978, 1980; Boobis & Chignell 1979; Glasson et al 1980; Tillement et al 1980).

As explanations the Gibbs-Donnan effect, unusual positive cooperativity, inhibition of binding by endogenous ligands and protein-protein interactions have been proposed. Of these, protein-protein interactions seem the likeliest, whilst several authors regard polymerization as an explanation of their findings (Scholtan 1962; Crawford et al 1972; Paubel & Niviere 1973; Müller & Wollert 1976; Zini et al 1976). Pedersen (1962) and Kolb & Weber (1975) have claimed that commercial albumin preparations contain significant amounts of polymers. Recently, Zini et al (1981) have reported on the concentration-dependent polymerization of a widely used human serum albumin (HSA). At a HSA concentration of 1 g litre^{-1}, 99% monomers were detected, but only 60% at physiological concentrations (40 g litre^{-1}). In contrast, it appears that only small amounts of HSA dimers, if any, and practically no other polymers are present in-vivo (Saifer et al 1961; Andersson 1966; Bocci 1967). To evaluate possible albumin polymerization and the presence of endogenous substances in serum on binding, we have compared the binding of metbufen, a new non-steroidal anti-inflammatory drug, to human serum, a commercial HSA, and an HSA sample isolated by us from normal human serum.

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Methods

Human serum (HSA = 600 μM), taken from healthy subjects and frozen, was used. Total proteins and free fatty acids (FFA) were determined (Sampson & Hensley 1975). The mole ratio of 6 FFA/HSA was 0·89. As a commercial source, HSA from Sigma Chemical Co. (Sigma A-1887, fatty acid free), was prepared in 0·067 M phosphate buffer and also stored at −30 °C. All serum or HSA dilutions were similarly made with phosphate buffer. Isolated HSA was prepared from serum via affinity chromatography using a sepharose gel (Zini et al 1981). The mole ratio of 6 FFA/HSA of this albumin was 0·55. [^{14}C]Metbufen (4-*p*-biphenylyl-2-methyl-4-oxo-butyric acid, 1·19 mCi mmol^{-1}, P. Fabre, France) was dissolved in methylpyrrolidone and dilutions made in phosphate buffer. The organic phase never exceeded 5%. Final metbufen concentrations were between 1 and 1500 μM in all cases except when binding to diluted serum (2–2500 μM). All experiments were performed with an equilibrium dialysis technique (Dianorm). Dialysis time was 2 h. The activity in 100 μl of both the dialysate and protein phase were counted in 15 ml of scintillation cocktail (quenching corrected by external standardization). Metbufen recovery after dialysis was always higher than 95%.

Binding was found to be saturable and the number of binding sites, n, and the affinity constant, K_a, were calculated according to their derivation from the law of mass action. The molarity of albumin was substituted for serum since albumin is the only binding protein. Parameters were first estimated via Rosenthal's (1967) linearization procedure and then refined by means of non-linear least squares approximation using a Gauss-Newton algorithm (Zini et al 1979). Binding percentages were calculated according to the formula

$$\%\,B = \frac{(B) - (A)}{(B)} \times 100 \qquad (1)$$